NEURONAL CELL PATTERNING ON COVALENTLY BOUND PROTEIN PATTERNS BY MICRO-CONTACT PRINTING TECHNIQUES AND THE FUNCTIONING OF PROTEINS BOUND ON SILANE MONOLAYERS

ChangJu Chun* and James J. Hickman
Nanoscience and Technology Center at University of Central Florida
Orlando, FL 32826

Weishi Wang, Cassie Gregory, Sriman Narayanan, and Matthew Poeta
Bioengineering Department at Clemson University
Clemson, SC 29634

ABSTRACT

Micro-patterning of neuronal cells *in vitro* is a critical step for studies in the fundamental biology of neuron-neuron and neuron-surface interactions. The culturing of neuronal cells on patterned self-assembled monolayers (SAMs) in some cases require further chemical modifications of the SAM surfaces to induce cell adhesion and promote neurite outgrowth. In these cases it would be important to select a negative surface modifying agent such as an extracellular matrix protein like tenascin-C and a specific functional protein like an antibody that interacts with neuronal cell adhesion molecules to support cell adhesion on specially designed surface patterns. The protein modified surfaces could then be used to arrange cells in specific patterns and control cell growth because of a specific protein function such as an inhibition of cell outgrowth. Protein immobilization on solid substrates could then be used as support layers for biosensors such as neuronal cell-based sensors, neuronal networks, biomedical devices, bioprocessing, bioassays, separations, and synthesis.

There is an increasing need in the field of biosensors to immobilize a functional protein on silica surfaces. Antibody immobilization on a silica surface can be easily accomplished by direct adsorption. However, this method results in partial denaturation of the protein, as well as an unstable attachment. Consequently, partial loss of protein can occur. Covalent attachments of functional proteins are one solution and are important when a coated substrate is subjected to a flowing solution or exposed for a long period of time in solution. Research indicates that covalent immobilizations of proteins can preserve their functions for up to two years.

In this study, we have developed a technique for protein patterning in two dimensions utilizing two proteins for *in vitro* studies of protein function. A combination of the self-assembled monolayer 3-mercaptopropyltrimethoxysilane (MTS) and then microcontact printing (μCP) of proteins using a patterned polydimethylsiloxane (PDMS) stamp has been used to create the protein patterns. The patterns were formed on the glass through several steps. First, a glass cover slip was silanized by MTS. Second, one end of the heterobifunctional crosslinker, N-succinimidyl 4-maleimidobutyrate (GMBS), formed a covalent link with a SH group of MTS, and the other end of GMBS was left for covalent binding of the protein. Third, antibody molecules were printed using the microcontact printing (MCP) method on the GMBS crosslinker surface to form patterns. The unpatterned crosslinker area was blocked by backfilling with a protein, tenascin-C, which inhibits neuronal growth. Through this method, protein patterns were achieved by covalent attachments on a glass surface. The non-specific protein adsorption problem was solved by backfilling with the blocking protein.

Hippocampal neurons were observed to grow axons and dendrites on the anti-NGF surfaces, which indicate that the antibodies immobilized using the crosslinking procedure could have retained their antigen-binding capability. The immobilized tenascin-C surface demonstrates that tenascin-C inhibits axonal growth while promoting dendrite outgrowth. Hippocampal neuronal patterns were formed on the immobilized anti-NGF / tenascin-C surface. The results summarized above can be helpful for further research into patterning neurons on an antibody surface through crosslinking to analyze the interactions between hippocampal neurons and antibodies or proteins on modified substrate surfaces.

1. INTRODUCTION

Patterning of surfaces with biological molecules is a method to develop biologically integrated devices with

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
00 DEC 2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Neuronal Cell Patterning On Covalently Bound Protein Patterns By Micro-Contact Printing Techniques And The Functioning Of Proteins Bound On Silane Monolayers	**g**
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Nanoscience and Technology Center at University of Central Florida Orlando, FL 32826; Bioengineering Department at Clemson University Clemson, SC 29634**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

resolutions from micron to the nanometer scale. Protein patterning is currently used for the development of biosensors for cell studies and tissue engineering applications. Several techniques, such as local deposition of molecules using microcontact printing methods, laser techniques, photochemical structuring, and photolithography (Blawas and Reichert, 1998; Kane et al., 1999; Lom et al., 1994; Ito, 1999; Clemence et al., 1998; Sorribas et al., 2002) have been examined to generate patterns of functional biomolecules on solid surfaces for applications such as neuronal cell-based biosensors and neuronal networks.

A method of protein immobilization on surfaces for creating protein patterns is physical adsorption where attraction between the solid surface and the protein results in coverage of the surface (Blawas and Reichert, 1998; Lin et al., 1998: Soderquist and Walton, 1980; Butler et al., 1993). Another method of protein immobilization is to covalently bind a protein to the surface using a chemical covalent bond (Sorribas et al., 2002; Chang, et al., 2003; Bhatia, et al., 1989; Pope, et al., 1993). Researchers in the protein patterning field have created micron level patterning in two dimensions using microcontact printing (μCP) with single proteins. We are interested in the development and application of techniques for protein patterning in two dimensions with two proteins per surface.

Micro-patterning of neurons *in vitro* is a critical step for studies in the understanding of the fundamental biology involved in neuron-neuron and neuron-surface interactions. A couple of investigators have developed patterned substrates to induce formation of neuronal networks from primary neuron culture (Liu, et al., 2000; Ma, et al., 1998; Dotti, et al., 1988; Kleinfeld, et al., 1988; Stenger, et al., 1998; Ravenscroft, et al., 1998). They have shown that chemically defined substrates can be used to control cell adhesion and neurite outgrowth.

The culturing of patterned neurons on proteins coupled to SAMs requires chemical modification of the SAM surface to induce cell adhesion and to promote neurite outgrowth. It is important to select specific substrates such as extracellular matrix proteins such as tenascin-C, and specific functional proteins, such as anti-NGF, that interact with neuronal cell adhesion molecules to support cell adhesion on designed surface patterns. We have examined technology to create cell patterns based on an initial surface of the 3-mercaptopropyl-trimethoxysilane (MTS) SAM combined with microcontact printing (μCP) of proteins from patterned polydimethylsiloxane (PDMS) stamps. These protein modified surfaces can be applied to create specific cellular patterns and control cell growth because of a specific protein function such as inhibition of cell outgrowth.

In this study, we have developed a technique for protein patterning in two dimensions with two proteins on a single surface for *in vitro* studies of protein function by using a combination of the self-assembled monolayer MTS, a heterobifunctional crosslinker and microcontact printing (μCP) of proteins with patterned PDMS stamps. We have determined that specific regions of proteins (anti-NGF) on the surface can be patterned to attach embryonic day 18 rat hippocampal neuronal cells. The remaining regions are backfilled with a protein, tenascin-C, which has been shown to be anti-adhesive for many cells and to promote cell rounding, but which also has been proposed to mediate neuron glia adhesion and to promote neurite outgrowth. (Jones and Jones, 2000; Chiquet-Ehrismann, et al., 1988; Fischer, et al., 1997).

2. MATERIALS AND METHODS

The MTS (Aldrich Chemical Company, Milwaukee, WI) solution was prepared in a glove box. The O_2 level in the glove box was less than 1ppm to prevent oxidation of the SH group of MTS. A volume of 125ml of distilled toluene was transferred to a beaker. Then, 2.5 ml of MTS was added to the toluene by pipette and mixed thoroughly to make a 2% MTS in toluene solution. Prior to coating, the glass coverslips were cleaned in a plasma cleaner (Harrick) at an oxygen pressure of 100 millitorr for 15 minutes. The cleaned glass cover slips were then immersed in the MTS toluene solution for one hour, rinsed in toluene three times, and allowed to air dry.

The N-succinimidyl 4-maleimidobutyrate cross-linker (GMBS) (Fluka) was dissolved in dry DMSO to make a 2mM GMBS in absolute ethanol solution. The MTS-coated cover slips were then immersed in the 2mM GMBS for one hour at room temperature.

After the reaction, the MTS-GMBS cover slips were washed with absolute ethanol three times. After being washed with PBS (1X), the MTS-GMBS cover slips were dried with N_2. Then the Anti-NGF (Chemicon, Temecula, CA) solution was applied to the Polydimethylsiloxane (PDMS) stamp and dried with a flowing stream of N_2. The anti-NGF was stamped on the cover slips for 2-5 minutes. After stamping, the cover slips were incubated at room temperature for more than two hours. Next, the cover slips were immersed in the blocking agent, tenascin-C (Chemicon International, Temecula, CA) solution and were backfilled for one hour. Finally, the cover slips were washed and rinsed with a 0.1% Tween 20 in PBS solution by shaking for 30 minutes, and then washed again with PBS three times.

Cell culture was in serum-free media containing neurobasal media, B-27 supplement and glutamine using embryonic day 18 rat hippocampal neuronal cells.

3. RESULTS AND DISCUSSIONS

Figure 1 depicts the reactions during the protein immobilization procedure. The thiol group on the silane reacts with the maleimide region of the heterobifunctional crosslinker, GMBS, by covalent binding for 1 hour. Then the succinimide residues bind terminal amino groups of the antibody, which was applied by microcontact stamping, or of tenascin-C, which was applied by immersion from a buffer solution.

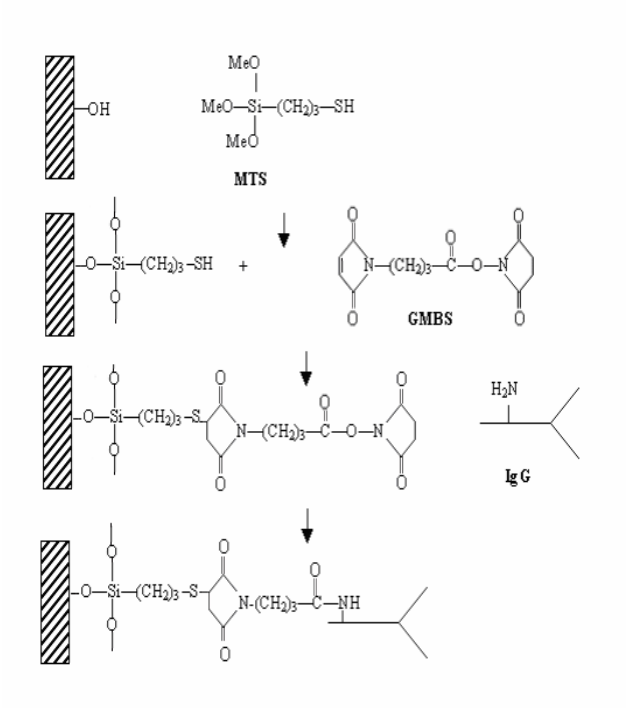


Figure 1: Schematic procedure of protein immobilization

The contact angles of the films were checked as a semi-quantitative guide to evaluate film quality and reproducibility. The cleaned glass cover slip contact angles were less than 5º before reacting with MTS. The contact angle of MTS coated coverslip was 55º+/-3º. The contact angle of the MTS-GMBS coverslips was approximately 45º.

Anti-NGF molecules were printed on the MTS-GMBS surface using a PDMS stamp, which contained a polarity pattern, as shown in Figure 2. The protein tenascin-C was backfilled on the unpatterned area to block the exposed GMBS succinimide residue. The procedure is indicated in Figure 3.

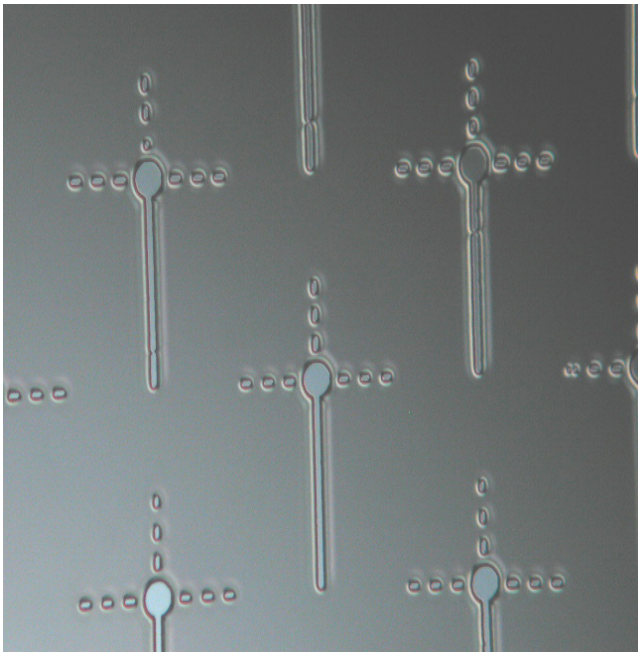


Figure 2: Optical image of the polarity pattern on the PDMS stamp (200X)

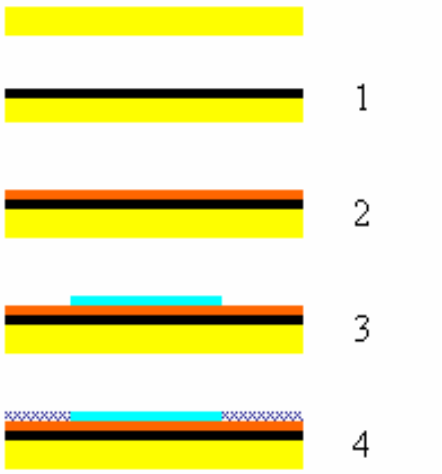
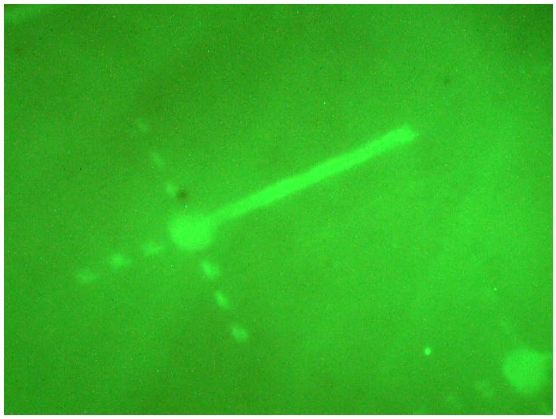
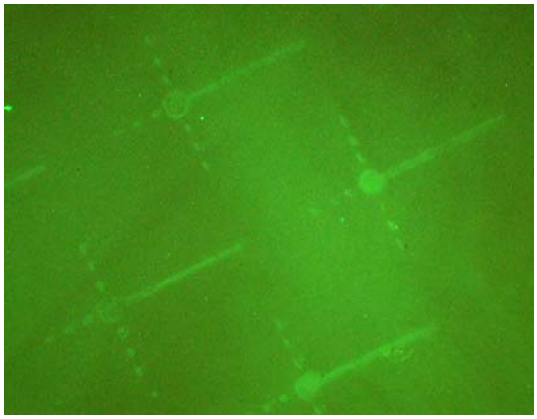


Figure 3: Illustration of pattern formation of protein immobilization 1) MTS silanization, 2) crosslinking GMBS, 3) microcontact printing of antibody, Anti-NGF 4) backfilling with a blocking protein, tenascin-C.

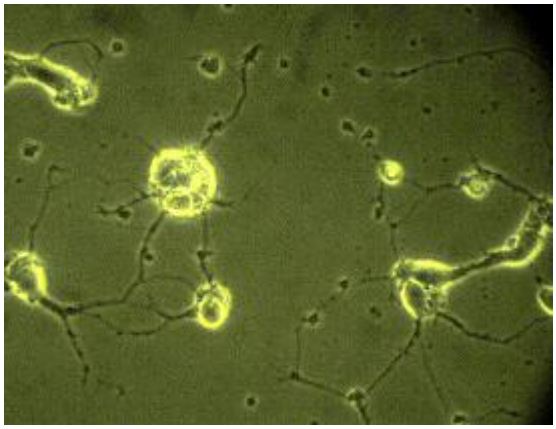


(a) 400X

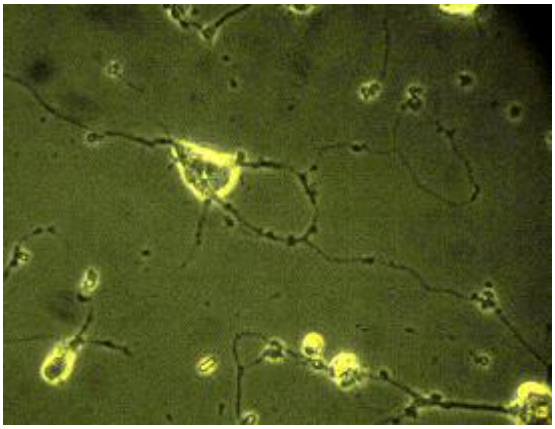


(b) 200 X

Figure 4: Micrographs of the anti-NGF polarity pattern then backfilled with tenascin-C. The green patterns were used to characterize the patterns by a secondary antibody reaction with anti-mouse IgG. X is the magnification.



(a) 400X



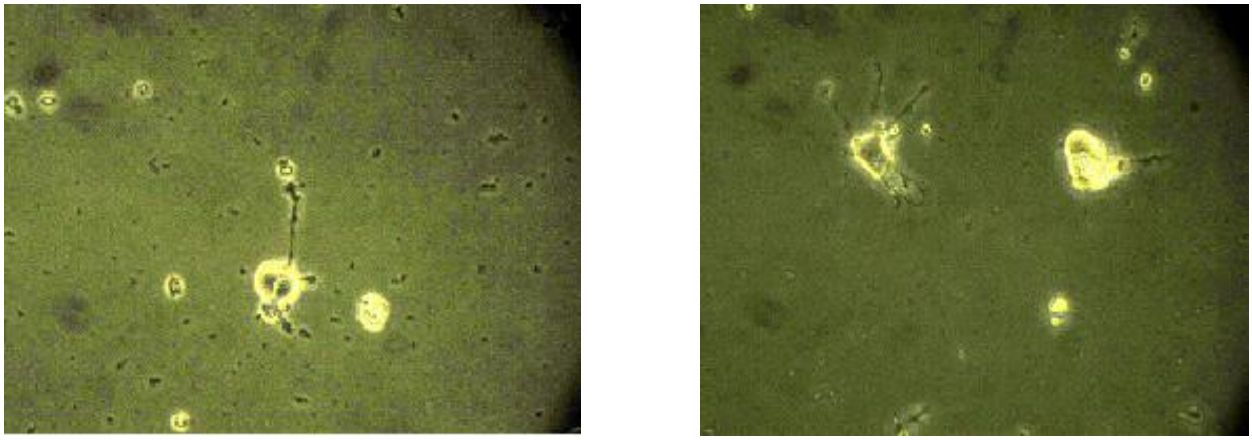
(b) 400X

Figure 5: Hippocampal neurons plated on an anti-NGF surface control without patterns. (a) and (b) in day 4 cultures

Before evaluating the samples by culturing neuronal cells, it was important to determine the existence of the protein pattern on the glass coverslips. Two glass coverslips were selected as samples for the secondary antibody reactions. Figure 4 indicates that an anti-NGF antibody polarity pattern (backfilling with tenascin-C) that was examined by a secondary antibody reaction using anti-mouse IgG. The picture was taken using a fluorescence microscope.

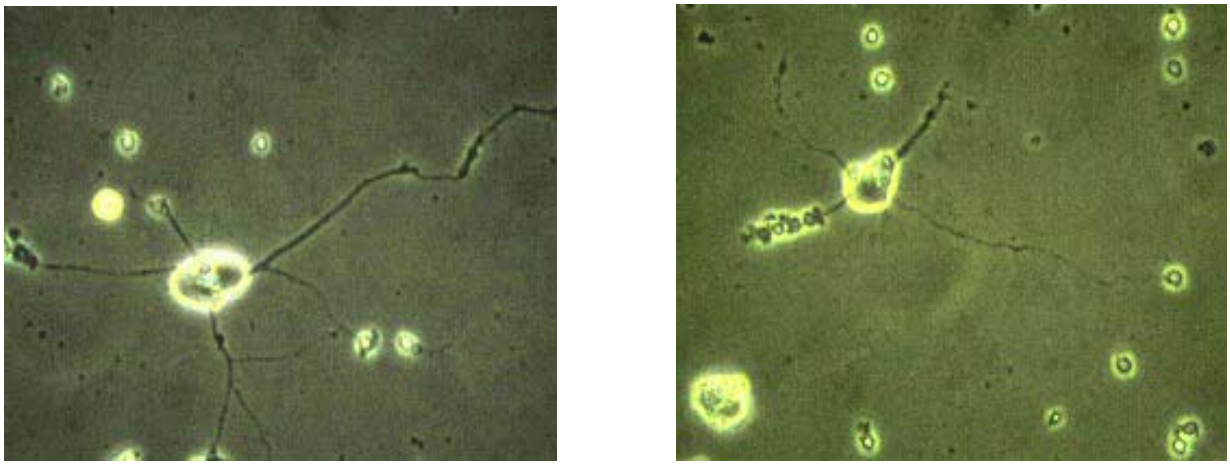
Two surfaces were then prepared for comparison of hippocampal neuronal cell growth. Anti-NGF and tenascin-C were immobilized on silanized glass coverslips through the GMBS crosslinker as indicated previously. Figure 5 shows neuronal growth on day 4 on an immobilized anti-NGF surface without patterns. Neuronal cells began to grow and extend processes. Nerve growth factor (NGF) receptor is a polypeptide that has a large effect on survival of peripheral and CNS neurons. In this research, anti-NGF was immobilized by a cross-linking reagent to a glass surface in an attempt to retain its antigen binding ability. Figure 5 shows that the anti-NGF surface promoted the hippocampal neuron attachment and neuronal cell growth on day 4 cultures.

Figure 6 shows that hippocampal neuron growth was limited on a tenascin-C surface on day 4, as only some cells had limited neurite outgrowth. Tenascin is a large glycoprotein that forms disulfide-linked hexamers. It has a multidomain with many repeat structural units including heptad, EFG-like and fibronectin type III repeats, as well as a homology to the globular domain of β- fibrinogen and γ-fibrinogen. Tenascin is expressed in distinctive spatial and temporal patterns in many embryonic tissues where tissue remodeling and cell migration occur. The tenascin-gene family consists of four distinct genes: Tenascin-C, Tenascin-R, Tenascin-X and Tenascin-Y. Tenascin-C is mainly expressed by glial cells during the development of the central and peripheral nervous system. The biological functions of tenascin are still partly controversial. Research indicates that the extracellular matrix glycoprotein tenascin-C has



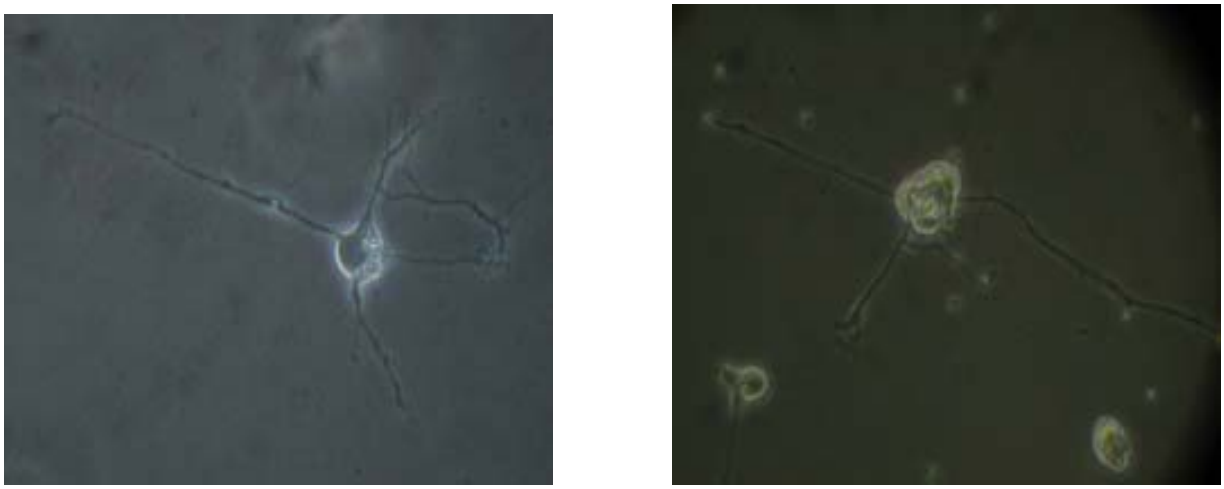
(a) 400X (b) 400X

Figure 6: Hippocampal neurons plated on a tenascin-C surface controls without patterns. (a) and (b) in day 4 cultures (morphologically immature processes)



(a) 400X (b) 400X

Figure 7: Patterned hippocampal neurons plated on an anti-NGF polarity pattern. The surface was backfilled with tenascin-C. (a) and (b) in day 3 cultures



(a) 400X (b) 400X

Figure 8: Patterned hippocampal neurons plated on the anti-NGF polarity pattern. The surface was backfilled with tenascin-C. (a) and (b) in day 7 cultures

the function of controlling cell adhesion, neuron migration and neurite outgrowth (Scholze, et al., 1996; Jones and Jones, 2000; Chiquet-Ehrismann, et al., 1988; Fisher, et al., 1997).

Tenascin-C has both stimulatory and inhibitory effects on CNS neurons. In some research, tenascin-C was used to study both extension and avoidance behaviors of growing axons and to demonstrate the dual aspects of astroglial effects on developing neuronal tissues (Faissner, et al., 1996). In this experiment, tenascin-C inhibited the hippocampal neurite outgrowth. The reason is not yet clear; however, it may be due to the distinct domains of tenascin-C, which underlies anti-adhesive (against cell-binding) for cells and promotes cell rounding for its inhibitory properties (Faissner, et al., 1990).

The results of culturing hippocampal neurons on an Anti-NGF and tenascin-C pattern demonstrated that on immobilized Anti-NGF, hippocampal neurons were observed to adhere on the surface. Tenascin-C had the ability to adhere hippocampal neurons but inhibited the neurite outgrowth. To pattern hippocampal neurons, anti-NGF was selected as the cell adhesion molecule while tenascin-C was chosen as the backfilling compound to inhibit the hippocampal neurite outgrowth. Hippocampal neurons were cultured on the patterned surfaces. Results were observed on day 3 and day 7, which are shown in Figure 7 and Figure 8. Hippocampal neurons grew and made neurites along the pathway of the polarity pattern. The results of the hippocampal neuronal pattern demonstrate that anti-NGF was the dominant factor for cell adhesion and neurite outgrowth on the anti-NGF/ tenascin-C patterns. Even though both tenascin-C and anti-NGF can promote hippocampal neuron adhesion, neuronal cells preferred to grow on the anti-NGF where neurite outgrowth was not inhibited.

By using the competitive properties of different proteins such as extracellular matrix proteins for neuron cell adhesion and growth, further study will focus on selecting and comparing different proteins for microcontact printing and backfilling for an optimal pattern formation of hippocampal neurons. The functionalities of the proteins are also very important after immobilizing on the substrates. Our further research will focus on keeping the natural functionality of proteins by using several high-affinity ligand pairs to immobilize proteins to substrates such as avidin-biotin, lectins, protein A and protein G, and a fragment of specific antibodies. We believe that the patterns of neuronal cells on the protein patterns are very useful for applications such as neuronal cell-based biosensors and neuronal networks in the near future.

4. CONCLUSION

Anti-NGF and tenascin-C were immobilized on glass substrates and tested for hippocampal neuronal cell growth. The antibody against NGF was observed to be a positive growth surface while tenascin-C demonstrated that it limited neurite outgrowth. Using the microcontact printing technique, with a subsequent backfilling step, anti-NGF patterns were formed on glass a substrate with a tenascin-C negative region. Patterned hippocampal neurons were achieved on the anti-NGF patterned surface.

ACKNOWLEDGMENTS

We would like to thank the Department of Energy for their support on this project, Grant Number DE-FG02-00ER45856.

REFERENCES

Bhatia, S.K., Shriver-Lake, L.C., Prior, K.J., Georger, J.H., Calvert, J.M., Bredehorst, R., and Ligler, F.S., 1989: Use of Thiol-Terminal Silanes and Heterobifunctional Crosslinkers for Immobilization of Antibodies on Silica Surfaces, *Analytical Biochemistry*, **178**, 408-413.

Blawas, A.S., and Reichert, W.M., 1998: Protein Patterning, *Biomaterials*, **19**, 595-609.

Buttler, J.E., Ni, L., Brown, W.R., Joshi, K.S., Chang, J., Rosenberg, B., and Voss, Jr. E.W., 1993: The Immunochemistry of Sandwich Elisas—VI. Greater Than 90% of Monoclonal and 75% of Polyclonal Anti-fluorescyl Capture Antibodies (CAbs) are Denatured by Passive Adsorption, *Molecular Immunology*, **30**, 1165-1175.

Chang, J.C., Brewer, G.J., and Wheeler, B.C., 2003: A Modified Microstamping Technique Enhances Polylysine Transfer and Neuronal Cell Patterning, *Biomaterials*, **24**, 2863-2870.

Chiquet-Ehrismann, R., Kalla, P., Pearson, C.A. Beck, K. and Chiquet, M., 1988: Tenascin Interfaces with Fibronectin Action, *Cell*, **53**, 383-390.

Clemence, J-F, Ranieri, J.P., Aebischer, P., and Sigrist, H., 1998: Photoimmobilization of a Bioactive Laminin Fragment and Pattern-guided Selective Neuronal Cell Attachment, *Bioconjugate Chem.*, **6**, 411-417.

Dotti, C.G., Sullivan, C.A., and Banker, G.A., 1988: The Establishment of Polarity by Hippocampal Neurons in Culture, *J. Neurosci.*, **8**, 1454-1468.

Faissner, A., Gotz, B., Joester, A., Wigger, F., Scholze, in Neural Pattern Formation and Plasticity, *Seminars in the Neurosciences*, **8**, 347-356.

Faissner, A. and Kruse, J., 1990: J1/Tenascin is a Repulsive Substrate for Central Nervous System Neurons, *J. Neuron*., **5**, 627-637.

Fischer, D., Brown-Ludi, M., Schulthess, T. and Chiquet-Ehrismann, R., 1997: Concerted Action of tenascin C Domains in Cell Adhesion, Anti-adesion and Promotion of Neurite Outgrowth, *J. Cell Sci*., **110**, 1513-1522.

Ito, Y. 1999: Surface Micropattering to regulate cell functions, *Biomaterials*, **20**, 2333-2342.

Jones, F.S. and Jones, P.L., 2000: The Tenascin Family of ECM Glycoproteins: Structure, Function, and Regulation during Embryonic Development and Tissue Remodeling, *Developmental Dynamics*, **218**, 235-259.

Kane, R.S., Takayama, S., Ostuni, E., Ingber, D.E., and Whitesides, G.M., 1999: Patterning Proteins and Cells Using Soft Lithography, *Biomaterials*, **20**, 2363-2376.

Kleinfeld, D., Kahler, K.H., and Hockberger, P.E., 1988: Controlled Outgrowth of Dissociated Neurons on Patterned Substrates, *J. Neurosci*., **8**, 4098-4120.

Lin, J.N., Andrade, J.D., and Chang, I-N., 1998: The Influence of Adsorption of Native and Modified Antibodies on Their Activity, *J. Immunol. Methods*, **125**, 67-77.

Liu, Q-Y., Coulombe, M., Dumm, J., Shaffer, K.M., Schaffner, A.E., Barker, J.L., Pancrazio, J.J., Stenger, D.A., and Ma, W., 2000: Synaptic Connectivity in Hippocampal Neuronal Networks Cultured on Micropatterned Surfaces, *Developmental Brain Research*, **120**, 223-231.

Lom B., Healy, K.E., and Hockberger P.E., 1994: A Versatile Technique for Patterning Biomolecules onto Glass Coverslips, *J. Neurosci. Meth*., **50**, 385-397.

A. and Schutte, K., 1996: Tenascin-C Glycoproteins

Ma, W., Liu, Q-Y., Jung, D., Manos, P., Pancrazio, J.J., Schaffner, A.E., Barker, J.L., and Stenger, D.A., 1998: Central neuronal synapse formation on micropatterned surfaces, *Developmental Brain Research*, **111**, 231- 243.

Pope, N.M., Kulcinski, D.L., Hardwick, A., and Chang, Y-A., 1993: New Application of Silane Coupling Agents for Covalently Binding Antibodies to Glass and Cellulose Solid Supports, *Bioconjugate Chem*., **4**, 166-171.

Ravenscroft, M.S., Bateman, K.E., et al., (1998). "Developmental Neurobiology Implications from Fabrication and Analysis of Hippocampal Neuronal Networks on Patterned Silane-Modified Surfaces," J. Amer. Chem. Soc. **120:**12169 (1998).

Scholze, A., Gotz, B. and Faissner, A., 1996: Glial Cell Interactions with Tenascin-C: Adhesion and repulsion to Different Tenascin-C Domains is Cell Type Related, *Int. J. Devl. Neuroscience*, **14**, 315-329.

Soderquist, M.E. and Walton, A.G., 1980: Structural Changes in Proteins Adsorbed on Polymer Surfaces, *J. Colloid Interface Sci.*, **75**, 386-397.

Sorrribas, H., Padeste, C., and Tiefenauer, L., 2002: Photolithographic Generation of Protein Micropatterns for Neuron Culture Applications, *Biomaterials*, **23**, 893-900.

Stenger, D.A., Hickman, J.J., Bateman, K.E., Ravenscroft, M., Ma, W., Pancrazio, J.J., Shaffer, K., Schaffner, A.E., Cribbs, D.H. and Cotman, C.W., 1998: Microlithographic Determination of Axonal/dentritic Polarity in Cultured Hippocampal Neurons, *J. Neurosci. Meth*., **82**, 167-173.

Stenger, D.A., Pike, C.J., Hickman, J.J. and Cotman, C.W., 1993: Surface Determinats of Neuronal Survival and Growth on Self-assembled Monolayers in Culture, *Brain Res*., **630**, 136-147.